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13. ABSTRACT (Maximum 200 Words)

The **purpose** of this proposal is to evaluate the ability of a replication-restrictive adenovirus (Ad-OC-E1a) to specifically target and lyse cells of an androgen independent prostate cancer osseous metastasis, which account for a majority of the morbidity and mortality experience by men with prostate cancer. The **scope** of this project to perform the studies outlined in proposal to prove the hypothesis that conditional replication under the guidance of the osteocalcin promoter can exert a prostate cancer-specific cell kill in well defined pre-clinical models of human androgen independent prostate cancer metastases. More specifically, Specific Aim I seeks to evaluate the specificity of the tumor-restrictive replication of Ad-OC-E1a using in vitro assays on prostate and non-prostate cancer cells. The ability of the Ad-OC-E1a to have at least a 100 fold killing differential favoring OC + cell lines (LNCaP, C4-2, PC-3) over OC- cell lines (LOVO, PrSC). Specific Aim II evaluates the growth inhibition of human prostate cancer xenografts attributable to Ad-OC-E1a administration, as well as, the tissue distribution and toxicity profile of such injections. The ability to Ad-OC-E1a to completely destroy both androgen-dependent and androgen-independent human prostate cancer cells in xenografts supports the hypothesis being studied.

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Introduction

The **subject** of this proposal is to develop and test the ability to a genetically modified common cold virus to destroy androgen-independent prostate cancer cells. Androgen-independent prostate cancer cells account for 100% of the mortality associated with prostate cancer. The **purpose** of this proposal is to evaluate the ability of a replication-restrictive adenovirus to specifically target and lyse cells of an androgen independent prostate cancer osseous metastasis. The **scope** of this project to perform the studies outline in the two specific aims to prove the hypothesis that conditional replication under the guidance of the osteocalcin promoter can exert a prostate cancer-specific cell kill in well defined pre-clinical models of human androgen independent prostate cancer metastases. More specifically, Specific Aim I seeks to evaluate the specificity of the tumor-restrictive replication of Ad-OC-E1a using in vitro assays on prostate and non-prostate cancer cells. Specific Aim 2 evaluates the growth inhibition of human prostate cancer xenografts attributable to Ad-OC-E1a administration, as well as, the tissue distribution and toxicity profile of such injections.

Body

Metatstatic prostate cancer remains a daily challenge for the urologist, oncologist and radiation oncologist. The relatively unique patophysiology underlying the formation of osteoblastic lesions predominately isolated to the bone of men with metastatic prostate cancer has allowed us to transcriptionally target these cancer cells with an osteoblastic promoter, osteocalcin. We have previously utilized a replication-defective adenovirus containing the osteocalcin promoter driving toxic gene expression to target osseous metastases in pre-clinical models and a phase I clinical trial (Ad-OC-TK). The transcription regulation of transgene expression using tumor- and tissue-specific promoters within adenoviral vectors has been shown to impart tumor or tissue specificity. The osteocalcin promoter has been demonstrated to effectively and safely target prostate cancer(1) and osteosarcoma(2-4) based on the shared osteoblastic phenotype, using a suicide gene therapy approach in preclinical and phase I testing(5).

The lytic replicative cycle of the adenovirus was initially used shortly after the discovery of the adenovirus for the treatment of cancer(6). The greater understanding of the adenoviral genetic make-up and function has led to the ability to construct conditionally replicating adenoviruses. Restrictive adenoviral replication has been used previously to target p53 mutated (7) cells and more recently PSA producing cells(8). In this paper we demonstrate that previously defined transcriptional specificity of the osteocalcin promoter can be used to destroy prostrate cancer cells by harnessing this adenoviral lytic replication cycle both in vitro and in vivo using relevant models of human hormone-refractory prostate cancer. This is achieved by placing the E1a gene under the transcriptional regulation of the osteocalcin promoter. By constructing an adenoviral vector that has the E1a gene under the control of the murine osteocalcin promoter the osseous metastases, which account for most of the morbidity and eventual mortality attributable to prostate cancer, can be effectively targeted.

Illustration 1 demonstrates the rational underlying this proposal. The osteocalcin promoter has the ability to transcriptionally regulate the production of the E1a protein in osteocalcin positive cell types. The production of this essential protein then directs adenoviral replication and eventual cell lysis. The lytic life cycle of the adenovirus is then allowed to proprogate throughout a tumor mass. The propagation wave will potentially continue until normal osteocalcin negative cells are encountered at the periphery of the tumor.
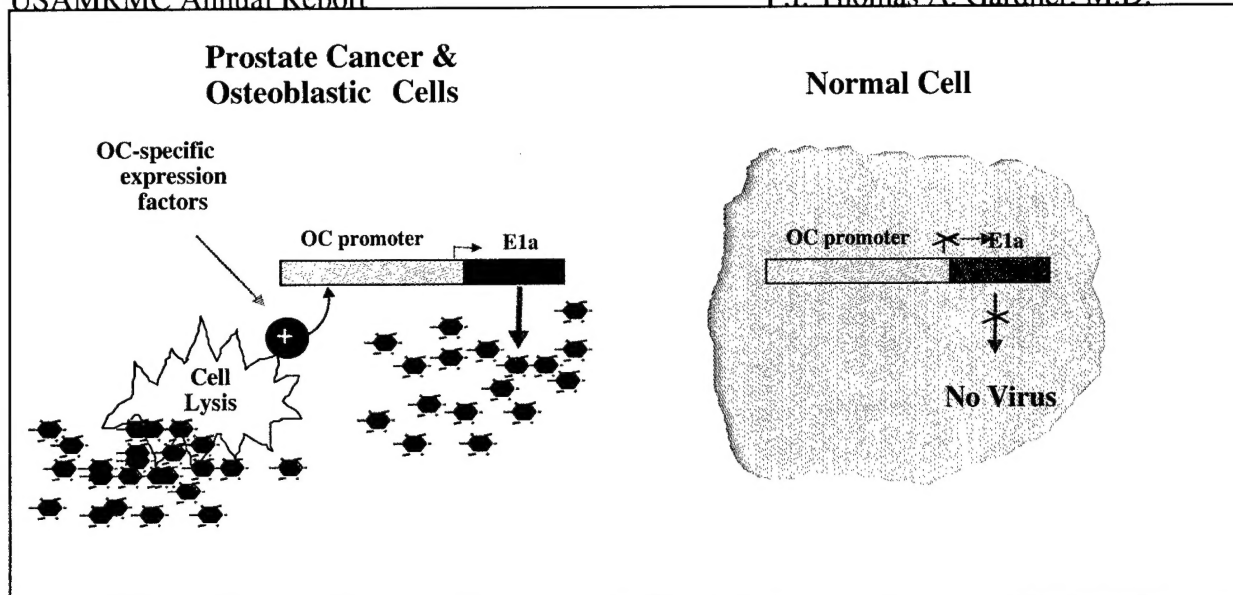
Illustration 1: The Rationale of Osteocalcin-restricted Adenoviral Oncolysis

The completion of the tasks outlined by the Statement of Work of the initial proposal is being performed with slight modifications of the timing as described below. In general, some of the In vivo studies projected for the second year of the proposal were initiated at the 6-month mark and results will be presented. This was a result of equipment difficulties that have been resolved that delayed the initiation of 3-dimensional studies. The research findings will be broken down by Task # as per the "Format Requirements for Preparing Reports".

Task 1 was to amplify, purify, quantify titer and confirm the activity of sufficient viral stocks of Ad-OC-E1a and Ad-CMV-Bgal. This task is ongoing and the PI and other members of the research team continue to improve on the technique in several ways. The PI and others have developed a novel production technique that allows for adenoviral production using a serum-free hollow fiber system that will be published in Biotechniques in 2001. Currently, sufficient Ad-OC-E1a and Ad-CMV-Bgal has been produced to perform the next 6 months of experiments. The adenoviral production continues on schedule. The anticipated problem of wild-type Ad5 was generated during the amplification process of Ad-CMV-B Gal requiring re-isolation of the virus and subsequent re-amplification. The occurrence delayed the completion of task 2.

Virus construction and production
The shuttle plasmid pOCE1a was constructed by starting with the shuttle vector p E1sp1B, provided by Dr. Frank Graham (McMasters University, Hamilton, Ontario, Canada). p E1sp1B contains the right end of the adenovirus type 5 genome, nucleotides 28 to 347, encoding several minor E1a promoters. To stop the transcription initiated from these minor E1a promoters, an SV40 polyadenylation signal (170 bp, Cla I – Hind III fragment, from pXCMVPA, obtained from Dr. Wei-Wei Chang) was cloned into p E1sp1B to generate p BE1sp1BPA. The Ad5 E1 region, from pX548c (also provided by Frank Graham), was cloned into p BE1sp1BPA. These subcloning procedures created the shutter vector p E1, which contains the 5'-end of the adenovirus type 5 genome, from nucleotides 28 to 347 and 549 to 5852, with multiple cloning sites between sequences 347 to 549. p E1 contains the majority of the E1 region except part of the E1a promoter, nucleotides 348 to 548. A mouse osteocalcin promoter (1370 bp, Not I-EcoR I fragment) from pII1.5, including TATA box, was cloned between the SV40 polyadenlyation site and E1a sequence of p E1 to generate **p OCE1a**, which has E1a under the transcriptional regulation of the osteocalcin promoter. [pII1.5 was provided by Dr. Gerard Karsenty of the

University of Texas M. D. Anderson Cancer Center, Houston Texas.] The sequence of p OCE1a was generated by known sequence information and analyzed by restriction enzyme digestion. The virus was generated using standard(9) and amplified using the standard and a hollow fiber production(10).

Task 2 was to perform DNA quantification and time course experiments with Ad-OC-E1a using dot blot experiments. These experiments are ongoing and final results from this task will be presented in the next annual report.

Task 3 and 4 was to perform in vitro killing assays in a variety of human cell lines. This task has been accomplished and the material, methods, results and discussion follow:

Methods and Materials

Cell lines and cell cultures:

The LNCaP cell line [Graham, 1977 #656] was kindly supplied by Dr. Gary Miller (University of Colorado, Denver, CO). C4-2 was established from LNCaP tumors propagated in castrated hosts(11,12). PC-3 (13)was obtained from the American Type Culture Collection (Rockville, MD). ROS 17/2.8 (ROS), a rat osteoblastic osteosarcoma cell line was generously supplied by Dr. Cindy Farrach-Carson (The University of Texas Dental Branch, Houston, TX). Prostate stromal cells (PrSC) were obtained from Clonetics (Walkersville, MD). LNCaP, C4-2, and PC-3 cell lines were maintained in T-medium [80% Dulbecco's modified Eagle's medium (DMEM; GIBCO, Grand Island, NY), 20% F12K (Irving Scientific, Santa Ana, CA), 3 gm/L $NaHCO_3$, 100 units/mL penicillin G, 100μg/ml streptomycin, 5 μg/ml insulin, 13.6 pg/ml triiodothyronine, 5 μg/ml transferrin, 0.25 μg/ml biotin, and 25 μg/ml adenine] with 10% fetal bovine serum (FBS; Sigma Chemical Company, St. Louis, MO). ROS cells were maintained in DMEM (GIBCO, Grand Island, NY) supplemented with penicillin (100 units/mL), streptomycin (100mg/ml), and 10% fetal bovine serum (FBS; Sigma Chemical Company, St. Louis, MO). PrSC cells were maintained in Stromal cell basal media, supplemented with the Stromal cell growth media BulletKit (Clonetics, Walkersville, MD). All cell cultures were maintained at 37° C in a humidified atmosphere of 95% air and 5% carbon dioxide. The cells were fed three times per week with fresh growth media.

MTT proliferation assay:

Cells were plated in 24 well plates at the following initial seeding densities in cells/well based on growth rates and prior experience in other in vitro assay on these cells: ROS (10,000), PC-3 (10,000) LNCaP (15,000), PrSC (15,000), C4-2 (40,000). Twenty-four hours after seeding, fresh media was placed on the cells and the cells were exposed to variable concentrations of the Ad-OC-E1a vector (0.01, 0.1, 1, 10, and 100 viral particles/cell; 4 wells each dilution) dissolved in PBS. An additional 4 wells that were treated with vector-free PBS served as controls. The media on all wells was changed every two days. Relative cell numbers were determined at intervals were by incubating the cells with MTT (thiazolyl blue) for 4 hours. Briefly, cells were then solubilized in a solution of 10% sodium dodecyl sulfate and 0.1 N hydrochloride solution for 16 hours. Absorbance was measured at wavelength 550 nm. The in vitro cell-killing activity of Ad-OC-E1a ranged from 0.01 to100 viral particles per cell was evaluated on an androgen-independent and metastatic human prostate cancer C4-2 cell line from 0 to 7 days.

Task 3 and 4 Results and Discussion:

The viral lytic effect of Ad-OC-E1a is demonstrated in LNCaP (OC-positive) cells at five day after infection with 1 vp/per cell. The left panel of Figure 1 demonstrates LNCaP cells five days after 1 vp/cell of Ad-CMV-B-Gal exposure. The right panel of Figure 1 demonstrates the

significant lytic ability of Ad-OC-E1 on LNCaP five days after 1 vp/cell exposure. The typical cytopathic effect (CPE) is seen in the right panel while absent in the left panel.
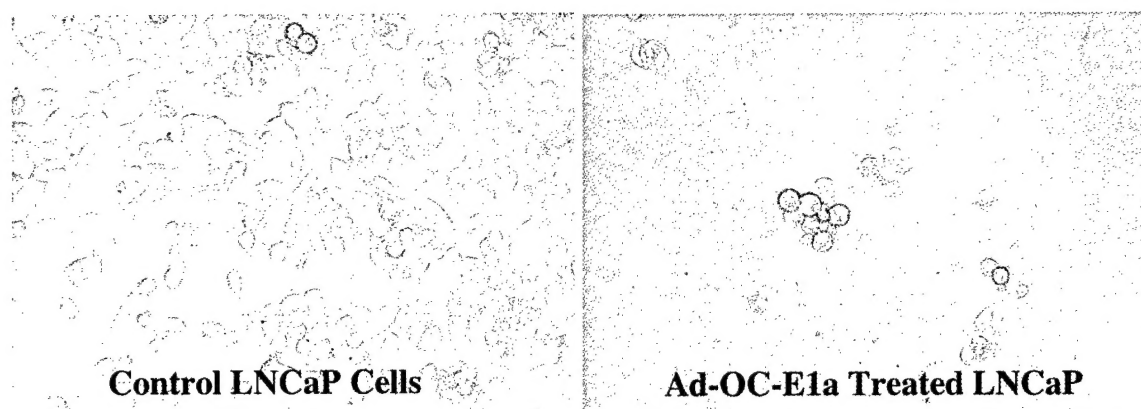

Control LNCaP Cells   **Ad-OC-E1a Treated LNCaP**

Figure 1. CPE 5 days after Exposure of LNCaP to Ad-OC-E1a (1 vp/cell)

The osteocalcin positive cell lines LNCaP, C4-2 and PC-3 all demonstrate a dose-dependent cell lysis as evaluated by MTT assay. The PrSC serve as a osteocalcin-negative relevant human cell line and demonstrates no cell lysis at day 7. The ROS cell line, expresses higher levels of osteocalcin, but serves a negative control for viral replication since the human adenovirus cannot replicate in rodent cells. (**Figure 2 below**)
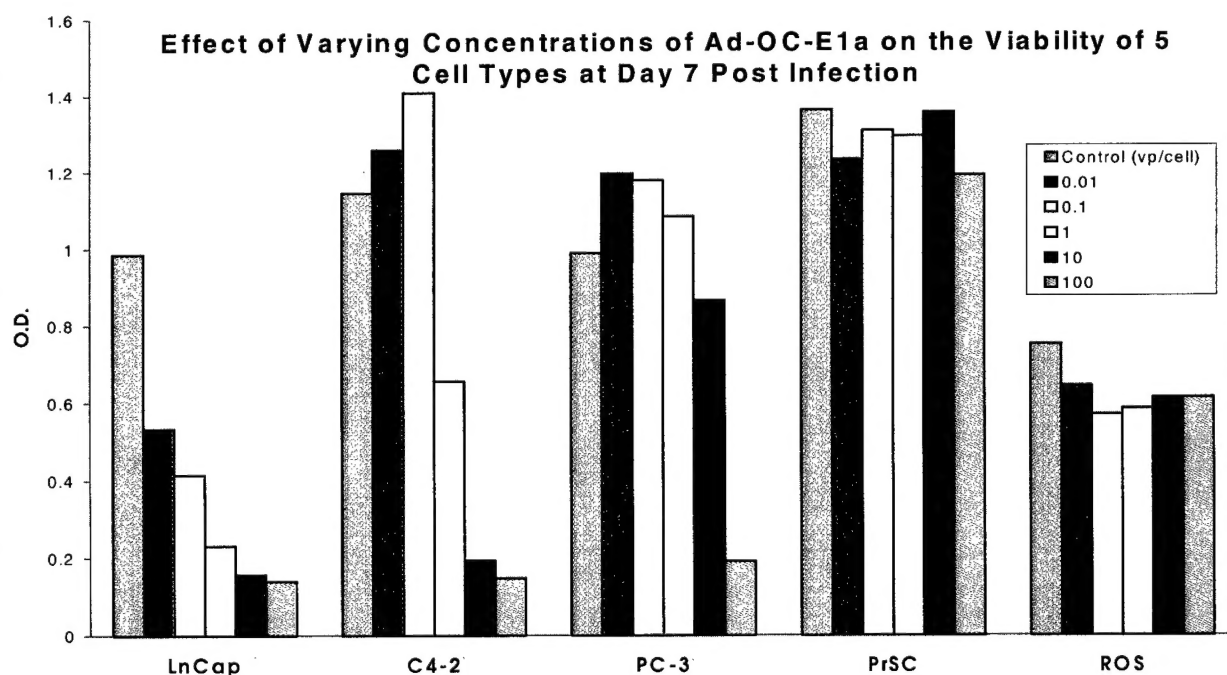

Figure 2: Osteocalin-Resistricted and Viral Particle-Dependent Cell Lysis by MTT Assay

Figures 3 and 4 demonstrate a time course of the Ad-OC-E1a dependent lysis in osteocalcin positive C4-2 cells and osteocalcin negative LOVO cells, respectively. A 100 fold differential is seen between the OC+ C4-2 cell and the OC- LOVO cells. Quantitative PCR will be performed on the DNA extracts from Days 0, 1, 3, 5, 7 to generate the viral production levels and time course.
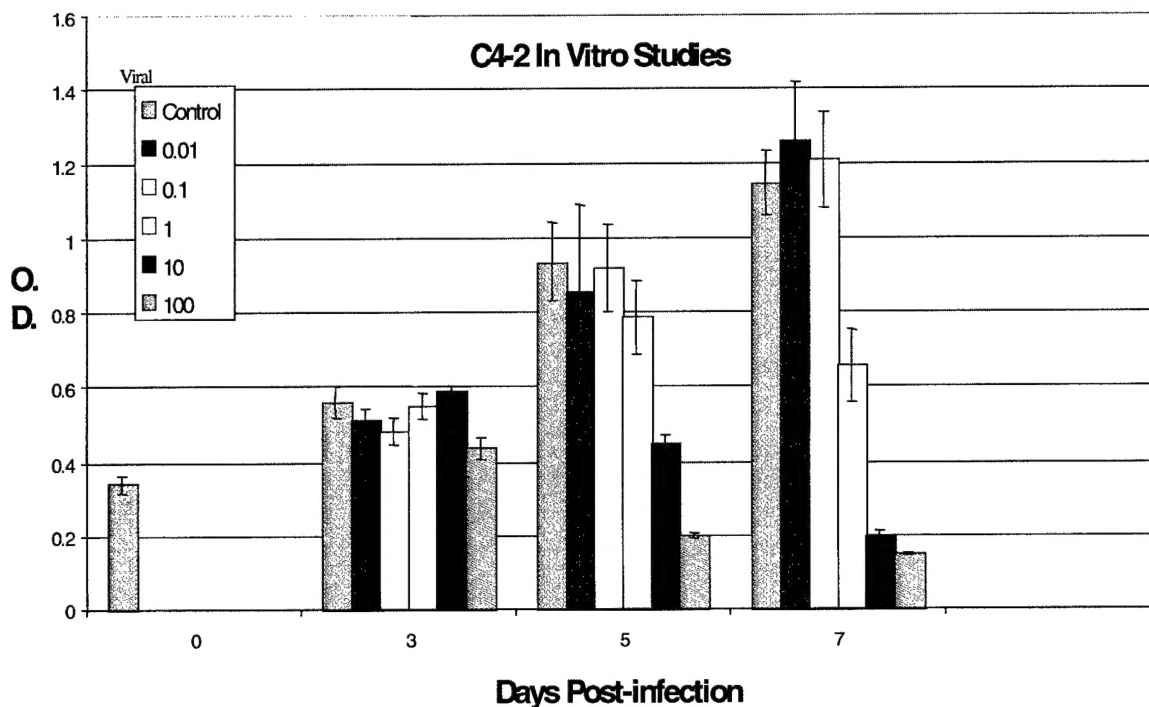

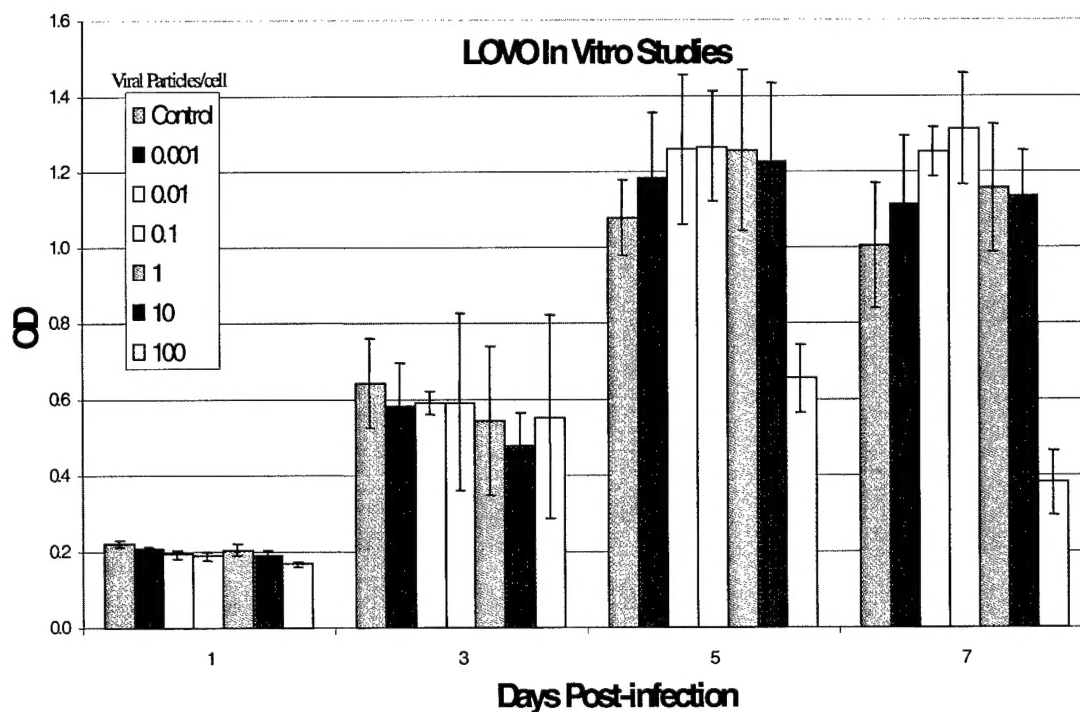**Figure 3: Time Course of Ad-OC-E1a Osteocalcin Dependent Cell Lysis in C4-2 Cells as Measured by MTT Assay**

Figure 4: Time Course of Ad-OC-E1a Osteocalcin Dependent Cell Lysis in LOVO Cells as Measured by MTT Assay

Task 5 is to analyze the results of the first 12 months to allow completion of this annual report. The Statistical analysis of this data presented in this report is being reviewed by the biostatistician of the cancer center and complete statistical analysis will be included in the next annual report.

Task 6 is to conduct the microgravity experiments to assess the lytic ability of Ad-OC-E1a on various human cancer organiods. This task is ongoing and not complete, therefore, the results will be included in next year's annual report. One unexpected occurrence was a malfunction of the microgravity chamber that required repair by the company and ultimately a new apparatus. I have just completed a test run of the new microgravity apparatus and it functioned well for a two-month experiment without malfunction. It is anticipated that the proposed completion of this task may be only slightly beyond the anticipated completion at 18 months.

Task 7 is to conduct subcutaneous xenograft experiments confirming the in vivo lytic activity post Ad-OC-E1a administration. This task is ongoing, therefore, the results will be presented in next year's annual report.

Task 8 is to evaluate the viral distribution time course and growth inhibition of intraosseous xenograft model. A portion of this task is completed, the methods, materials, results and discussion follow:

Methods and Materials: Intraosseous Xenografts with C4-2
Using an intraosseous model of androgen-independent prostate cancer, C4-2 cells were directly inoculated in the tibia or femur of nude mice and serum PSA was followed until greater 5 ng/dl. (12). Mice bearing these intraossoeous xenografts were then treated with intralesional administration of Ad-OC-E1a of a control reporter virus Ad-CMV-B-Gal 1×10^9 pfu per lesion on one occasion. These animals were followed with weekly serum PSA's, radiographic findings and necropsy at 10 weeks post injection.

Results and Discussion Task 8
Figure 5 demonstrates the establishment of stable intraosseous xenografts using the C4-2 model. The y-axis is the serum PSA in ng/dl with each bar representing one mice on Day 0 of the intralesional study. The seven bars on the left received Ad-OC-E1a injections and the seven bars on the right received Ad-CMV-B-Gal injections. The starting PSAs were comparable in each group.
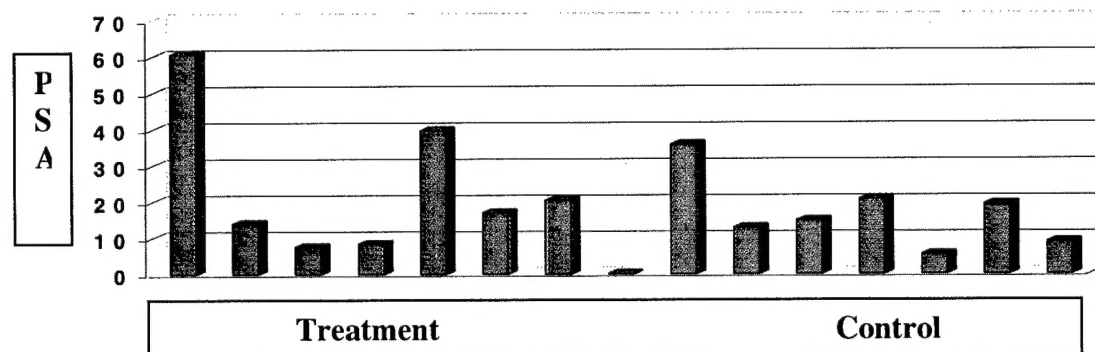

Figure 5 Day 0 Serum PSA (ng/dl) in Intraosseous C4-2 Model

Figure 6 and 7 show the weekly PSA readings for the control and treatment group, respectively. As predicted the control animals have increasing PSA values until sacrifice despite receiving Ad-

CMV-B-Gal injections on day 0. Note that the scale of the y-axis on a 0-1000 scale while the treatment group is on a 0-100 scale to avoid dwarfing the treatment group's non-existent PSA values.
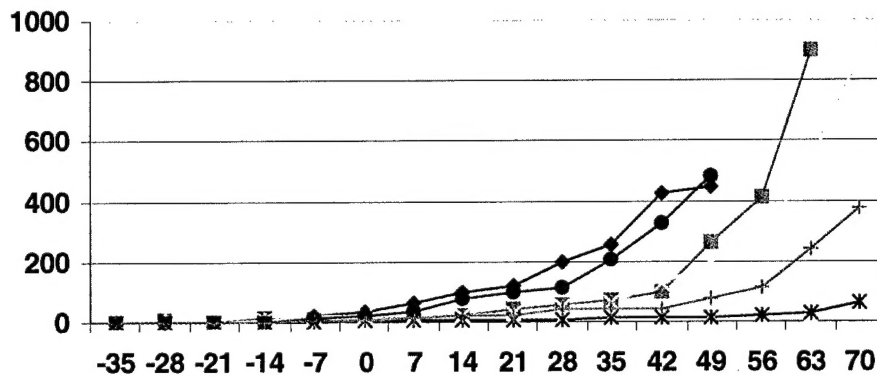

Figure 6: Serum PSA (ng/dl) of Immunocompromised Mice with Intraosseous C4-2 after One Intralesional Ad-CMV-B-Gal (1 x 10^9 PFU) on Day 0.
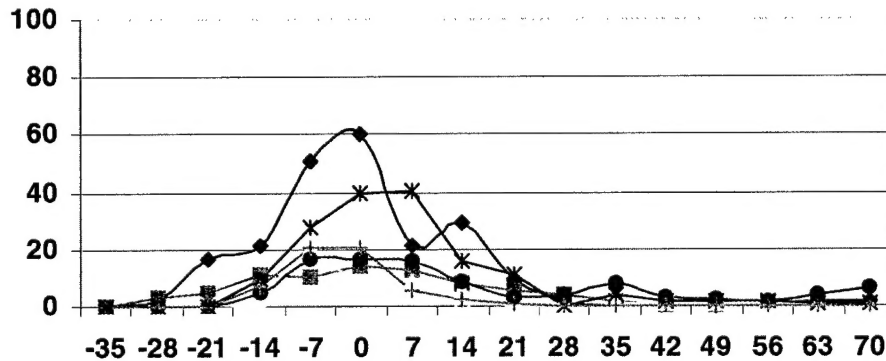

Figure 7: Serum PSA (ng/dl) of Immunocompromised Mice with Intraosseous C4-2 after One Intralesional Ad-OC-E1a (1 x 10^9 PFU) on Day 0

Figure 8 represents radiographs at sacrifice of one control (left) and one treated (right) mice, respectively. These radiographs illustrate the significant growth inhibition of intraosseous C4-2 tumors as evidenced by both PSA decline and normalization of radiograph after intralesional Ad-OC-E1a. Note the large tumor on the tibia of the control animal and the normal radiographic picture of the mouse after receiving the injection of Ad-OC-E1a.

Task 9 is to examine the histopathological correlations with viral distribution study of task #8. This examination is ongoing and results will be reported in next year's annual report.

Task 10 is to analysis the results prior to submission of the 2nd annual report.   This task will be complete by the submission deadline for the 2nd annual report.

Task 11 is to conduct the viral distribution after a variety of delivery techniques. This task is not schedule to start until month 24 and has not begun

Task 12 is the final data compilation, statistical analysis, manuscript preparation and final report preparation. The results to date can be broken up into in vitro and in vivo findings will be submitted to Molecular Therapy with the title being **"Osteocalcin promoter-based adenoviral oncolytic obliteration of human prostate cancer metastatic models."**

Key Research Accomplishments
- **Confirmation of In Vitro Specificity of OC promoter in OC+ Cell lines.**

- **Confirmation of reproducibility of C4-2 Intraosseous Model**

- **Confirmation of Intraosseous Tumor growth inhibition after intralesional injection.**

Reportable Outcomes

Gardner TA, Wada Y, Shirakawa T, Ko S-C, Kao C, Kim SJ, Yang L, Chung LWK. Osteocalcin promoter restricted adenoviral replication as a potential treatment of prostate cancer metastasis. Presented at the 8th International Conference on Gene Therapy of Cancer, San Diego, CA, December 1999. **1999 Vical Best Abstract Award**

Gardner TA, Wada Y, Sukay M, Yang L, Brown L, Ko S-C, Cheng L, Chung LWK, Kao C. Osteocalcin promoter-based adenoviral oncolytic obliteration of human prostate cancer metastatic models. Molecular Therapy (submitted 1/2001).

Gardner TA, (PI) OBA Gene Transfer Protocol #0010-426 "Phase I study of intratumoral injections of OCaP1(Ad-OC-E1a) for metastatic or locally recurrent prostate cancer, Part 1: Dose finding, Part 2: Index lesion escalation"

Conclusions
This proposal is designed to test the hypothesis the adenoviral lytic replication cycle can be placed under the transcriptional regulation of the osteocalcin promoter. Since the initiation of the work the osteocalcin promoter continues to perform well in osteoblastic diseases such as osteosarcoma and prostate cancer in both pre-clinical and clinical settings. The work described above further illustrates the specificity of this promoter. The results to date can be simply divided into in vitro and in vivo results.   In the in vitro assays demonstrate at a 100 fold better cell kill in the OC+ (LNCaP, C4-2, PC-3) and OC- (LOVO and PrSC). The completion of these in vitro experiments supported the early investigation in the in vivo setting. The in vivo finding of near-complete abrogation of PSA in mice with established intraosseous C4-2 tumors compared to controls also provides strong evidence to support the current hypothesis under investigation. In summary, the proposal is being carried out close to the time line proposed with

significant findings having already been discovered. These findings are already being brought to the clinic in the phase I trial of Ad-OC-E1a for men with metastatic prostate.

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